PEPTIDE HAVING FOR FIBRINOGEN FRAGMENT E ACTIVITY, ANALOGS, ANTIBODIES AND USES THEREOF

Field of the Invention.

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The present invention relates broadly to compounds which act as modulators or mimics of fibrin fragment E and their use.

Introduction

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Basic Pathology of Chronic Inflammation, Healing and Repair:

Fibrinogen is the major circulating plasma protein involved in blood clot formation. Activation of the clotting enzyme

15 cascade, by for example injury or inflammation, results in conversion of prothrombin to thrombin which cleaves two small fibrinopeptides (A and B) from each soluble fibrinogen molecule to give fibrin monomer. Cross linkage of monomers is the final step of the coagulation system that gives solid

20 fibrin. Whole blood includes platelets and forms blood clot in wounds, and is termed thrombus when in abnormal arteries and veins: inflammatory exudate is platelet free and forms fibrin alone.

25 Fibrin deposition and degradation is a major feature of the pathology of acute and chronic inflammation at any site in the body regardless of the underlying disease aetiology. This process is apparent at the histological level in the healing wound, the organising thrombus, the advanced atherosclerotic

30 plaque, and many other types of pathological lesions including the growing edge of some types of cancer. The fibrin mesh provides a provisional matrix for cell ingrowth, being progressively invaded in wound healing by inflammatory cells (macrophages), new small blood vessels (capillary buds),

35 connective tissue cells (fibroblasts) and the epidermis (squamous epithelium). In the context of the large arteries subject to atherosclerosis, the endothelium of the luminal surface and the smooth muscle cells of the vascular wall

invade the fibrin mesh. Secretion of plasminogen activator is
the common factor that provides controlled lysis of the fibrin
substratum via plasmin degradation, releasing fibrin
degradation products. Fibrin degradation products are
5 composed of combinations of two moieties termed fragments D
and E. Eventually the fibrin present is replaced by new cells
and matrix forming new tissue. These basic features apply to
many types of human and animal disease.

10 **Fibrin Degradation Products:**

Although fibrin may be a factor common to many pathologies
involving cell proliferation, it has generally been assumed
that its main function was to provide an inert physical matrix
15 to support cell movement. However there has been evidence for
some time that fibrinopeptides and fibrin degradation products
have biological activity particularly as soluble mediators of
chemotaxis, the phenomenon of directional cell movement (1).
It has been proposed that fibrin degradation products were a
20 major pathological growth factor common to all sites of
chronic inflammation. Using the chick chorioallantoic
membrane as an in vivo test model for detection of angiogenic
growth factors it has been suggested that fibrin degradation
products were angiogenic (2), had stimulatory effects on
25 collagen synthesis (3), and that specifically fibrin fragment
E may be the active component (4).

Fibrin deposition and lysis is believed to be relevant to a
wide spectrum of human diseases including vascular restenosis,
30 cancer, atherogenesis, rheumatoid arthritis, diabetes and
renal diseases.

US 5 981 697 describes generation of antibodies which bind
fibrinogen fragments E1, E2 and E3. Bach et al (1998) J Biol
35 Chem 273 pp30719 - 30728 describes an interaction of the
fibrinogen β15-42 sequence with endothelial cell VE-cadherin.
Lee et al (1999), Molecules and Cells 9(1) 7-13 describes the

stimulation of production of IL-6 in macrophages by fragment E of fibrin and fibrinogen.

Summary of the invention.

The production of antibodies to fibrin fragment E has been previously described, the antibodies being derived using an Fd phage combinatorial library of random epitope display to select clones binding and common to both polyclonal rat and rabbit anti E blocking antisera (32). In the present invention, the inventors have individualised a number of compounds which modulate the induction of cell proliferation induced by fibrin degradation products. The present inventors have further demonstrated that fibrin fragment E binds to a cell membrane component of approximately 66kDa in size using, for example, ligand blotting of SDS-PAGE gels of cell membranes from chick fibroblasts. The presence of a specific binding site for fragment E raises the possibility that agonists and antagonists of its binding could be used to modulate its binding and thus modulate its effects. Such effects include induction of cell proliferation, angiogenesis, fibrogenesis and collagen synthesis. Such agonists and antagonists, such as those disclosed herein, may also have modulatory effects on, for example, cell stimulation per se. Such modulation may be very useful in the control of cell proliferation seen in atherosclerosis, and particularly in post angioplasty restenosis. Using the techniques described below, the inventors have identified a number of compounds which modulate the FDP induced stimulation of cell proliferation. Preferably the variant retains fibrin fragment E activity.

Accordingly, the present invention provides a peptide comprising a sequence selected from the group consisting of:

CRAHSFGSPRPLPVV   (SEQ ID NO:1)
SRAHSFGSPRPLPVV   (SEQ ID NO:2)

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CRAHSFVSPRPLPVV   (SEQ ID NO:3)
QPDPHLMMWKLPGFP   (SEQ ID NO:4)

or a fragment thereof capable of modulating fibrin fragment E
5   activity.

As described above, fibrin fragment E activity refers to at
least one of following activities: induction of cell
proliferation, angiogenesis, fibrogenesis and collagen
10   synthesis.

In a further aspect of the invention, there is provided a
functional variant of the above peptide, which variant
comprises from one to four, preferably from one to three, more
15   preferably one or two, amino acid variations, including
substitutions, insertions and deletions. Preferably, the
variant retains the capability of modulating fibrin fragment E
activity.

20   In another aspect of the invention there is provided a fusion
peptide which comprises a first portion having the amino acid
sequence of a peptide according to the invention as defined
above and a second portion, attached to the N- or C- terminus
of the first portion, which comprises a sequence of amino acid
25   not naturally contiguous to the first portion. Such
heterologous peptide fusions are also referred to herein as
peptides of the invention.

In a further aspect, the invention provides assay methods for
30   the identification of substances which bind to or modulate the
activity of peptides of the invention, either in monomeric or
oligomeric form.

In a further aspect of the invention, "analogs" of the
35   peptides of the invention are provided. Analogs are non-
peptide compounds which share fibrin fragment E activity, for
example the ability to competitively inhibit binding of FDPs ,

for example, fibrin fragment E to the fragment E receptor.
Analogs are a further aspect of the invention inasmuch as they
are novel. Analogs may be produced by any of the techniques
described herein or may be derived using, for example,
5   combinatorial chemical libraries known in the art. Examples
of such libraries are reviewed in Newton GR, Exp. Opin. Ther.
Patents (1997) 7(10): 1183-1194. Where the term "chemical
library" is used herein, those skilled in the art will
understand its meaning accordingly.
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The invention also provides antibodies and binding fragments
thereof capable of selectively binding to peptides or analogs
of the invention.

15 In a further aspect, the invention provides a pharmaceutical
composition comprising a peptide, analog or antibody according
to the invention together with a pharmaceutically acceptable
carrier or diluent.

20 Peptides, analogs or antibodies and compositions of the
invention may be used for a number of purposes. They are also
useful as research agents to investigate the receptor for
fibrin fragment E and the activation of cell proliferation by
fibrin degradation products. They may also be useful in
25 modulating fibrin fragment E activity such as cell
proliferation, particularly inhibition of angiogenesis.

Thus in a preferred aspect the peptides, analogs or antibodies
of the invention may be used in a method of modulating fibrin
30 fragment E activity, said method comprising introducing an
effective amount of a peptide of the invention. The method
may be practised *in vitro* or *in vivo*. Where it is practised
in vivo the invention will find use in a method of treatment
of the human or animal body, particularly in methods of
35 treating cancer or other proliferative diseases, including
restenosis, e.g. caused by regrowth of vascular cells
following angioplasty procedures.

Detailed Description of the Invention

Peptides

5   "Peptides" of the invention should be understood to include
fragments thereof and variants of said sequences and fragments
as set out above, as well as heterologous fusions of said
peptides.

10   The term "comprising" means "including" and allows for the
presence of further amino acid sequences, or other chemical
moieties, at the - and/or C-termini, provided that the peptide
as a whole retains the ability to bind to a fibrin fragment E
binding site.

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It is preferred that peptides of the invention (including
fragments of SEQ ID NOs 1-4 above and other sequences of the
invention described herein), excluding any heterologous fusion
sequence, are from 3 to 15, such as from 5 to 15, e.g. 5 to 8,
20   5 to 10, 8 to 15 or 8 to 11 amino acids in length.

The presence of a heterologous fusion sequence, which in
itself has no activity in binding to the fibrin fragment E
binding site will vary according to its intended function.
25   Such functions include the ability to translocate across
membranes, an epitope function to allow for purification or
identification of the peptide, and the like. As a rough
guide, the heterologous fusion sequence will be from 4 to 500,
such as from 4 to 100 or 10 to 50, e.g from 10 to 30 amino
30   acids in size.

The second peptide portion can be any sequence selected by
those of skill in the art taking into account the intended
purpose of the fusion. For example, the second portion may
35   comprise a detectable tag such as a T7 tag, HA tag or a myc
tag allowing identification of the peptide in a cell and/or
its recovery. The second portion may also be a signal

sequence directing expression of the peptide from a host cell
in which the fusion is being expressed. This will be useful
for the recombinant production of peptides of the invention.

5   The second portion may also comprise a molecular tag which
     influences the overall structure. A number of helix
     initiators which aid the formation of α-helixes which comprise
     short peptide sequences are known in the art.

10   In a preferred embodiment the second peptide portion is a
     membrane translocation sequence, capable of directing a
     peptide through the membrane of a eukaryotic cell. Example of
     such peptides include the HSV-1 VP22 protein (Elliot *et al*,
     1997), the HIV Tat protein (for example residues 1-72, 37-72
15   or 48-60; Fawell *et al*, 1994) or a sequence that is derived
     from the *Drosophila melanogaster* antennapedia protein, e.g.
     the 16 amino acid peptide sequence:
     Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-
     Lys.
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     A translocation peptide may be at the N-terminus or the C-
     terminus of the heterologous fusion.
     Unless the context requires otherwise, reference below to
     peptides of the invention includes the fusion peptides
25   described above.

     Variant Peptides

     Certain amino acid residues of peptides 1-4 above may be
30   substituted without significant loss of the ability of the
     peptide to bind to the fibrin fragment E binding site. Thus,
     amino acids of these peptides may be substituted to provide
     variant peptides which form a further aspect of the invention,
     within the above-described ranges.
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     Substitutions may include conserved substitutions, for example

according to the following table, where amino acids on the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R H
AROMATIC		F W Y

Alternatively, any amino acid may be replaced by a small aliphatic amino acid, preferably glycine or alanine.

In addition, deletions and insertions may also be made. Insertions are preferably insertions of small aliphatic amino acids, such as glycine or alanine, although other insertions are not excluded.

Variant peptides may also be modified in any of the ways described herein for peptides of the invention. This includes for example "reverse" C-terminal to N-terminal sequences, synthetic amino acids, modified side chains and labelling.

Where methods for the production and use of peptides of the invention are described, it will be understood that reference is also being made to variant peptides of the invention unless the context explicitly indicates otherwise.

In another aspect, a peptide of the invention may be provided in the form of molecules which contain multiple copies of the peptide (or mixtures of peptides of the invention). For example, the amino group of the side chain of lysine may be used as an attachment point for the carboxy terminus of an amino acid. Thus two amino acids may be joined to lysine via

carbonyl linkages, leading to a branched structure which may in turn be branched one or more times. By way of example, four copies of a peptide of the invention may be joined to such a multiple antigen peptide (MAP), such as a MAP of the

5 structure $Pep_4-Lys_2-Lys-X$, where Pep is a peptide of the invention (optionally in the form of a heterologous fusion), Lys is lysine and X is a terminal group such as β-alanine which provides for joining of the MAP core to a solid support such as a resin for synthesis of the Pep_4-MAP peptide and which

10 may be removed from the support once synthesis is complete.

Linear multimers of peptides of the invention may also be provided.

15 Other multiple peptide structures may be obtained using the MAP cores described in: Lu et al, 1991, Mol Immunol, 28, 623-30; Briand et al, 1992, J Immunol Methods, 156, 255-65; Ahlborg, 1995, J Immunol Methods, 179, 269-75.

20 A multimer of peptides of the present invention may be fused to a translocation peptide for directing it through the membrane of a eukaryotic cell, as discussed herein. A translocation peptide may be fused to an N-terminus or a C-terminus of the multimer, or it may be incorporated at an

25 intermediate position within the multimer.

Where multimers of the invention are provided, they may comprise different peptides of the invention or be multimers of the same peptide.

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Production and Modification of Peptides.

Except where specified to the contrary, the peptide sequences described herein are shown in the conventional 1-letter code

35 and in the N-terminal to C-terminal orientation. The amino acid sequence of peptides of the invention may also be modified to include non-naturally-occurring amino acids or to

increase the stability of the compound *in vivo*. When the
compounds are produced by synthetic means, such amino acids
may be introduced during production. The compound may also be
modified following either synthetic or recombinant production.

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Peptides of the invention may be made synthetically or
recombinantly, using techniques which are widely available in
the art. Synthetic production generally involves step-wise
addition of individual amino acid residues to a reaction
10   vessel in which a peptide of a desired sequence is being made.

Peptides of the invention may also be made synthetically using
D-amino acids. In such cases, the amino acids will be linked
in a reverse sequence in the C to N orientation. This is
15   conventional in the art for producing such peptides.

A number of side-chain modifications for amino acids are known
in the art and may be made to the side chains of peptides of
the present invention. Such modifications include for
20   example, modifications of amino groups by reductive alkylation
by reaction with an aldehyde followed by reduction with $NaBH_4$,
amidination with methylacetimidate or acylation with acetic
anhydride.

25   The guanidino groups of arginine residues may be modified by
the formation of heterocyclic condensation products with
reagents such as 2,3-butanedione or glyoxal. Sulphydryl
groups may be modified by methods such as carboxymethylation,
tryptophan residues may be modified by oxidation or alkylation
30   of the indole ring and the imidazole ring of histidine
residues may be modified by alkylation.

The carboxy terminus and any other carboxy side chains may be
blocked in the form of an ester group, e.g. a C_{1-6}alkyl ester.
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The above examples of modifications to amino acids are not
exhaustive. Those of skill in the art may modify amino acid

side chains where desired using chemistry known per se in the art.

Expression Vectors, Nucleic Acids and Host Cells

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In another aspect, the invention provides nucleic acids encoding peptides of the invention. For example, the nucleic acid may be a nucleic acid primer consisting essentially of between about 15 to 50 nucleotides. Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors.

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Such vectors may be transformed into a suitable host cell to provide for expression of a peptide of the invention. Thus, in a further aspect the invention provides a process for preparing peptides according to the invention which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions to

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provide for expression by the vector of a coding sequence
encoding the peptides, and recovering the expressed peptides.

The vectors may be for example, plasmid, virus or phage
5 vectors provided with an origin of replication, optionally a
promoter for the expression of the said polynucleotide and
optionally a regulator of the promoter. The vectors may
contain one or more selectable marker genes, for example an
ampicillin resistance gene in the case of a bacterial plasmid
10 or a neomycin resistance gene for a mammalian vector. Vectors
may be used *in vitro*, for example for the production of RNA or
used to transfect or transform a host cell.

A further embodiment of the invention provides host cells
15 transformed or transfected with the vectors for the
replication and expression of polynucleotides of the
invention. The cells will be chosen to be compatible with the
said vector and may for example be bacterial, yeast, insect or
mammalian.
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Promoters and other expression regulation signals may be
selected to be compatible with the host cell for which the
expression vector is designed. For example, yeast promoters
include S. cerevisiae GAL4 and ADH promoters, S. pombe nmt1
25 and adh promoter. Mammalian promoters include the
metallothionein promoter which is can be included in response
to heavy metals such as cadmium. Viral promoters include the
SV40 large T antigen promoter, retroviral LTR promoters and
adenovirus promoters. All these promoters are readily
30 available in the art.

The vector may also be adapted to be used *in vivo*, for example
in a method of therapy. Vectors suitable for use in therapy
include adenoviral vectors, retroviral vectors and alphavirus
35 vectors. Such vectors are adapted for use in therapy by a
number of modifications, for example by making the vector
replication defective. Reference may be made to, for example,

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WO95/14091 for a description of retroviral vectors and
WO95/07994 for a description of alphavirus vectors. The
disclosures of both references are hereby incorporated by
reference.

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Vectors for use in therapy will generally be administered in
the form of packed viral particles containing the vector, the
particles being delivered to the site of fibrin fragment E
activity , for example a tumour or other proliferating cells.

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Vectors for production of peptides of the invention or for use
in gene therapy include vectors which carry a mini-gene
sequence of the invention.

15 For further details see, for example, Molecular Cloning: a
Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold
Spring Harbor Laboratory Press. Many known techniques and
protocols for manipulation of nucleic acid, for example in
preparation of nucleic acid constructs, mutagenesis,
20 sequencing, introduction of DNA into cells and gene
expression, and analysis of proteins, are described in detail
in Current Protocols in Molecular Biology, Ausubel et al.
eds., John Wiley & Sons, 1992.

25 **Antibodies**

A peptide or analog according to the present invention may be
used as an immunogen or otherwise in obtaining specific
antibodies. Antibodies are useful in purification and other
30 manipulation of peptides or analogs, diagnostic screening and
therapeutic contexts. This is discussed further below.

The provision of the peptides or analogs of the invention also
enables for the production of antibodies which may bind the
35 portion of fibrin fragment E which interacts with its receptor
in a specific manner. Thus the invention provides an antibody
which is able to bind specifically to a peptide or analog of

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the invention or such portion.   Such antibodies may be
produced using epitopes of peptides or analogs of the
invention.

5   Another feature of the present invention is the generation of
anti-fragment E antibodies which prevent the binding of the
fibrin fragment E to its receptor. Polyclonal antibodies
which block the cell proliferative activity of fibrin fragment
E can be raised by injection of the whole fragment E in
10   rabbits. When admixed with fibrin degradation products the
cell proliferative activity is abolished (17).

For instance, purified peptides of the invention, or a variant
thereof, e.g. produced recombinantly by expression from
15   encoding nucleic acid therefor, may be used to raise
antibodies employing techniques which are standard in the art.
Antibodies and peptides or analogs comprising antigen-binding
fragments of antibodies may be used as discussed further
below.
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Methods of producing antibodies include immunising a mammal
(e.g. human, mouse, rat, rabbit, horse, goat, sheep or monkey)
with the protein or a fragment thereof. Antibodies may be
obtained from immunised animals using any of a variety of
25   techniques known in the art, and might be screened, preferably
using binding of antibody to antigen of interest (which may be
labelled). This is discussed below.

For instance, Western blotting techniques or
30   immunoprecipitation may be used (Armitage et al, 1992, Nature
357: 80-82). Antibodies may be polyclonal or monoclonal.

Modification of Antibodies

35   Antibodies may be modified in a number of ways. Indeed the
term "antibody" should be construed as covering any specific
binding substance having a binding domain with the required

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specificity. Thus, this term covers antibody fragments,
derivatives, functional equivalents and homologues of
antibodies, including any peptide comprising an immunoglobulin
binding domain, whether natural or synthetic. Chimaeric

5   molecules comprising an immunoglobulin binding domain, or
equivalent, fused to another peptide are therefore included.
Cloning and expression of Chimaeric antibodies are described
in EP-A-0120694 and EP-A-0125023. It has been shown that
fragments of a whole antibody can perform the function of

10   binding antigens. Examples of binding fragments are (i) the
Fab fragment consisting of VL, VH, CL and CH1 domains; (ii)
the Fd fragment consisting of the VH and CH1 domains; (iii)
the Fv fragment consisting of the Vl and VH domains of a
single antibody; (iv) the dAb fragment (Ward, E.S. et al.,

15   Nature 341, 544-546 (1989) which consists of a VH domain; (v)
isolated CDR regions; (vi) F(ab')2 fragments, a bivalent
fragment comprising two linked Fab fragments (vii) single
chain Fv molecules (scFv), wherein a VH domain and a VL domain
are linked by a peptide linker which allows the two domains to

20   associate to form an antigen binding site (Bird et al,
Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-
5883, 1988); (viii) bispecific single chain Fv dimers
(PCT/US92/09965) and (ix) "diabodies", multivalent or
multispecific fragments constructed by gene fusion

25   (WO94/13804; P Holliger et al Proc. Natl. Acad. Sci. USA 90
6444-6448, 1993).

Diabodies are multimers of peptides, each peptide comprising a
first domain comprising a binding region of an immunoglobulin

30   light chain and a second domain comprising a binding region of
an immunoglobulin heavy chain, the two domains being linked
(e.g. by a peptide linker) but unable to associate with each
other to form an antigen binding site: antigen binding sites
are formed by the association of the first domain of one

35   peptide within the multimer with the second domain of another
peptide within the multimer (WO94/13804).

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As an alternative or supplement to immunising a mammal, antibodies with appropriate binding specificity may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda

5   bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

Binding of peptides and analogs to antibodies

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Immunoassays for detecting antibodies are well known in the art and will generally comprise:

(a)   providing a peptide comprising an epitope bindable by an antibody against said protein;

15   (b)   incubating a biological sample with said peptide under conditions which allow for the formation of an antibody-antigen complex; and

(c)   determining whether antibody-antigen complex comprising said peptide is formed.

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A peptide or analog of the invention may be labelled with a revealing label. The revealing label may be any suitable label which allows the peptide or analog to be detected. Suitable labels include radioisotopes, e.g. ^{125}I, enzymes,

25   antibodies, polynucleotides and linkers   such as biotin. Labelled peptides or analogs of the invention may be used in diagnostic procedures such as immunoassays in order to determine the amount of a peptide or analog of the invention in a sample. Peptides or analogs or labelled peptides or

30   analogs of the invention may also be used in serological or cell mediated immune assays for the detection of immune reactivity to said peptides or analogs in animals and humans using standard protocols.

35   A peptide or analog or labelled peptide or analog of the invention or fragment thereof may also be fixed to a solid phase, for example the surface of an immunoassay well or

dipstick.

Such labelled and/or immobilized peptides or analogs may be
packaged into kits in a suitable container along with suitable
5   reagents, controls, instructions and the like.

Such peptides or analogs and kits may be used in methods of
detection of antibodies to such peptides or analogs present in
a sample or active portions or fragments thereof by
10 immunoassay.

Uses of Antibodies

Antibodies raised to a peptide can be used in the
15 identification and/or isolation of variant peptides, and then
their encoding genes. Thus, the present invention provides a
method of identifying or isolating a fibrin degradation
product binding epitope or variant thereof (as discussed
above), comprising screening candidate peptides with a peptide
20 comprising the antigen-binding domain of an antibody (for
example whole antibody or a fragment thereof) which is able to
bind said fibrin degradation product binding epitope peptide
or variant thereof, or preferably has binding specificity for
such a peptide. Specific binding members such as antibodies
25 and peptides comprising antigen binding domains of antibodies
that bind and are preferably specific for a fibrin degradation
product binding epitope peptide or mutant or derivative
thereof represent further aspects of the present invention, as
do their use and methods which employ them.
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Candidate peptides for screening may for instance be the
products of an expression library created using nucleic acid
derived from an animal of interest, or may be the product of a
purification process from a natural source. Analogs may be
35 produced by any of the techniques described herein or may be
derived using, for example, combinatorial chemical libraries
known in the art. Examples of such libraries are reviewed in

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Newton GR, Exp. Opin. Ther. Patents (1997) 7(10): 1183-1194.

A peptide found to bind the antibody may be isolated and then
may be subject to amino acid sequencing. Any suitable
5   technique may be used to sequence the peptide either wholly or
partially (for instance a fragment of the peptide may be
sequenced). Amino acid sequence information may be used in
obtaining nucleic acid encoding the peptide, for instance by
designing one or more oligonucleotides (e.g. a degenerate pool
10 of oligonucleotides) for use as probes or primers in
hybridization to candidate nucleic acid, or by searching
computer sequence databases, as discussed further below.

The reactivities of antibodies with a sample may be determined
15 by any appropriate means. Tagging with individual reporter
molecules is one possibility. The reporter molecules may
directly or indirectly generate detectable, and preferably
measurable, signals. The linkage of reporter molecules may be
directly or indirectly, covalently, e.g. via a peptide bond or
20 non-covalently. Linkage via a peptide bond may be as a result
of recombinant expression of a gene fusion encoding antibody
and reporter molecule.

The mode of determining binding is not a feature of the
25 present invention and those skilled in the art are able to
choose a suitable mode according to their preference and
general knowledge.

Antibodies according to the present invention may be used in
30 screening for the presence of a peptide or analog, for example
in a test sample containing cells or cell lysate as discussed,
and may be used in purifying and/or isolating a peptide or
analog according to the present invention, for instance
following production of the peptide by expression from
35 encoding nucleic acid therefor.

Antibodies may modulate the activity of the peptide or analog

to which they bind and so, if that peptide or analog has a
deleterious effect in an individual, may be useful in a
therapeutic context (which may include prophylaxis).

5   An antibody may be provided in a kit, which may include
instructions for use of the antibody, e.g. in determining the
presence of a particular substance in a test sample. One or
more other reagents may be included, such as labelling
molecules, buffer solutions, elutants and so on. Reagents may
10   be provided within containers which protect them from the
external environment, such as a sealed vial.

Development of Mimetics

15   A peptide, analog or antibody according to the present
invention may be used in screening for molecules which bind to
it or modulate its activity or function. Such molecules may
be useful in a therapeutic (possibly including prophylactic)
context.
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The stimulation of cell proliferation induced by fibrin
degradation products, for example fibrin fragment E, provides
a target for the development of therapeutic agents capable of
inhibiting uncontrolled cell proliferation, for example found
25   in restenosis or in tumour cells.

A number of assay formats are described in WO94/10307 and
WO96/10425. The provision of the peptides, analogs or
antibodies of the invention provide positive control reagents
30   for such assays which will be desirable in the design of high
throughput screening assays for novel compounds which can
exert a similar effect. The peptides, analogs or antibodies
of the invention further provide a basis for rational drug
design of pharmaceutical compounds to target the fibrin
35   fragment E binding site.

Peptides, analogs or antibodies of the present invention may

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be used to develop mimetics. This might be desirable where
the peptide, analog or antibody is difficult or expensive to
synthesise or where it is unsuitable for a particular method
of administration, e.g. peptides may be unsuitable active
5   agents for oral compositions as they may be quickly degraded
by proteases in the alimentary canal. Mimetic design,
synthesis and testing may be used to avoid randomly screening
large numbers of peptides or analogs for a target property.

10 There are several steps commonly taken in the design of a
mimetic from a peptide, analog or antibody having a given
target property. Firstly, the particular parts of the
peptide, analog or antibody that are critical and/or important
in determining the target property are determined. In the
15 case of a peptide, this can be done by systematically varying
the amino acid residues in the peptide, e.g. by substituting
each residue in turn. These parts or residues constituting
the active region of the peptide are known as its
"pharmacophore".

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Once the pharmacophore has been found, its structure is
modelled according to its physical properties, e.g.
stereochemistry, bonding, size and/or charge, using data from
a range of sources, e.g. spectroscopic techniques, X-ray
25 diffraction data and NMR. Computational analysis, similarity
mapping (which models the charge and/or volume of a
pharmacophore, rather than the bonding between atoms) and
other techniques can be used in this modelling process.
A template molecule is then selected onto which chemical
30 groups which mimic the pharmacophore can be grafted. The
template molecule and the chemical groups grafted on to it may
conveniently be selected so that the mimetic is easy to
synthesise, is likely to be pharmacologically acceptable, and
does not degrade in vivo, while retaining the biological
35 activity of the lead peptide, analog or antibody.
Alternatively, the pharmacophore can be used to form the basis
of a search of a computer database of structures to identify a

mimetic. The mimetic or mimetics found by any approach described herein can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation and/or modification can then be carried

5 out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Mimetics obtainable by the above and other methods available in the art form a further aspect of the present invention.

10

Compositions

Peptides, analogs or antibodies of the invention may be in a substantially isolated form. It will be understood that the

15 peptide, analog or antibody may be mixed with carriers or diluents which will not interfere with the intended purpose of the peptide, analog or antibody and still be regarded as substantially isolated. A peptide, analog or antibody of the invention may also be in a substantially purified form, in

20 which case it will generally comprise the peptide, analog or antibody in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the peptide, analog or antibody in the preparation is a peptide, analog or antibody of the invention.

25 Peptides or analogs of the invention may be formulated in the form of a salt. Salts of peptides or analogs of the invention which may be conveniently used in therapy include physiologically acceptable base salts, eg derived from an appropriate base, such as alkali metal (e.g. sodium), alkaline

30 earth metal (e.g. magnesium) salts, ammonium and NR_4 (wherein R is C_{1-4} alkyl) salts. Salts also include physiologically acceptable acid addition salts, including the hydrochloride and acetate salts.

35 Peptides, including fusion peptides, analogs, or antibodies of the invention may be formulated into pharmaceutical compositions. The compositions comprise the peptide, analog

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or antibody together with a pharmaceutically acceptable
carrier or diluent. Pharmaceutically acceptable carriers or
diluents include those used in formulations suitable for oral,
topical, or parenteral (e.g. intramuscular or intravenous)
5   administration. The formulations may conveniently be
presented in unit dosage form and may be prepared by any of
the methods well known in the art of pharmacy. Such methods
include the step of bringing into association the active
ingredient with the carrier which constitutes one or more
10 accessory ingredients. In general the formulations are
prepared by uniformly and intimately bringing into association
the active ingredient with liquid carriers or finely divided
solid carriers or both, and then, if necessary, shaping the
product.

15

For example, formulations suitable for parenteral
administration include aqueous and non-aqueous sterile
injection solutions which may contain anti-oxidants, buffers,
bacteriostats and solutes which render the formulation
20 isotonic with the blood of the intended recipient; and aqueous
and non-aqueous sterile suspensions which may include
suspending agents and thickening agents, and liposomes or
other microparticulate systems which are designed to target
the peptide, analog or antibody to blood components or one or
25 more organs.

Suitable liposomes include, for example, those comprising the
positively charged lipid (N[1-(2,3-dioleyloxy)propyl]-N,N,N-
triethylammonium (DOTMA), those comprising dioleoyl-
30 phosphatidylethanolamine (DOPE), and those comprising 3β[N-
(n',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol).

Compositions may comprise any desired amount of a peptide,
analog or antibody of the invention. In part this will depend
35 upon the intended formulation and its intended use. By way of
general guidance the composition may comprise from about 1% to
about 99%, for example from 10% to 90% of a peptide, analog or

23

antibody of the invention.

The composition may comprise a mixture of more than one, for example two or three, peptides, analogs or antibodies of the invention.

Peptides, analogs or antibodies of the invention may also be used in conjunction with a second agent capable of inhibiting cell proliferation, in order to provide a combined anti-proliferative effect. Thus the composition may also comprise other pharmaceutically active ingredients, in particular cytotoxic and/or cytostatic agents.

Alternatively, a peptide, analog or antibody of the invention may be delivered to a patient in a separate composition from a cytotoxic or cytostatic agent but simultaneously or sequentially. "Sequentially" means that one of the peptides, analogs or antibodies or the agent will be delivered first, and the other delivered within a period of time such that the enhanced effect of the two agents together is achieved in a target proliferating cell. Where one or both agents is delivered over a period of time, e.g. through intravenous infusion, the time period of administration of the agents may be sequential or overlapping.

When used in methods of treatment of the human or animal body, the peptide, analog or antibody and the agent may be administered to a subject at the same site or at different sites.

Thus the invention provides a peptide, analog or antibody of the invention and a cytotoxic or cytostatic agent for separate or simultaneous use in the treatment of proliferating cells, for example tumour cells, either *in vitro* or *in vivo*.

Where *in vitro* use is contemplated, this will include *ex-vivo*, e.g. in the treatment of bone marrow from a subject which may

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be reimplanted into the subject after treatment.

The invention further provides the use of a peptide, analog or antibody of the invention for the manufacture of a medicament for the treatment of proliferating cells wherein said cells are also treated, separately or simultaneously, with a cytotoxic or cytostatic agent.

Numerous cytotoxic and/or cytostatic agents are known in the art (e.g. listed in The Merck Index, 12th Edition, 1996) and include:

alkaloids such as etoposide and other toposiomerase inhibitors, paclitaxel, vinblastine and vincristine;

alkylating agents such as alkyl sulphonates (e.g. busulfan), aziridines, ethylenimines and methylmelomines (e.g. triethylenemelamine and triethylenephosphoramide), nitrogen mustards (e.g. cyclophosphamide, melphalan and uracil mustard), nitrosoureas and the like;

antibiotics and analogues such as actinomycins, anthramycin, doxorubicin, puromycin and the like;

antimetabolites such as folic acid analogues (e.g. methotrexate), purine analogues (e.g. 6-mercaptopurine and thioguanine) and pyrimidine analogues (e.g fluorouracil);

platinum complexes such as cisplatin; and

other anti-neoplastic compounds including for example hydroxyurea.

In addition, the cytotoxic or cytostatic compound may be an immunomodulatory compound or hormonal analogue compound. Examples of the former include interferons α, β and γ and interleukins such as IL-2. Examples of the latter include antiandrogens, antiestrogens (e.g. tamoxifen), aromatase inhibitors, estrogen analogues, LHRH analogues (e.g. buserelin) and the like.

Cytostatic compounds also include antimetastatic agents such as matrix metalloproteinase inhibitors such as batimastat.

Methods of Treatment

Peptides, analogs, or antibodies of the invention may also be used in methods of treating a number of diseases, in
5   particular those diseases in which uncontrolled proliferation of cells may play a part. Conditions in which uncontrolled cell proliferation may be treated include vascular restenosis, cancer, atherogenesis, rheumatoid arthritis, diabetes and renal diseases and psoriasis.
10

Angiogenesis and Wound Healing

Fibrin deposition and lysis are essential features, as demonstrated by abnormalities of healing in the plasminogen
15   and fibrinogen knockout mouse models (5, 6). The present inventors have shown in a mouse incised wound model that the peak of angiogenic activity in simple wound extracts occurs at day 3, preceding the peak of wound vascular density at day 5 (7) with the bulk of the angiogenic activity suggested to be
20   attributable to fibrin fragment E (8). Compounds of the invention may therefore find use in treatments concerned with wound healing.

Vascular Restenosis

25

The likelihood of clinically significant post-angioplasty restenosis has been generally understood to be predicted to a fair extent by the amount of blood clot at the angioplasty
30   site. The antithrombin drug hirudin has been partially successful in reducing restenosis experimentally and in man (9), but it has been unclear whether this is attributable to clot reduction or prevention of direct thrombin stimulation of smooth muscle cell proliferation via the PAR-1 thrombin
35   receptor (10). However it is now apparent that the PAR-1 knockout mouse is normal and has near normal wound healing (11), and that intimal hyperplasia is not inhibited by an

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antisense thrombin receptor oligodeoxynucleotide following
carotid injury in the rabbit (12). An alternative candidate
that is both thrombin and plasmin dependant, but not thrombin
receptor dependant is fibrin fragment E. Fibrin degradation
products are abundant at sites of healing and repair,
including sites of vascular injury and in extracts of
proliferative types of human atherosclerotic plaque. The
present inventors have shown that fibrin fragment E stimulates
smooth muscle cell proliferation and outgrowth from aortic
media explants in culture (13). This occurs in serum rich
culture in which thrombin is inactive. The peptides, analogs
and antibodies therefore find use in treatment and prevention
of restenosis.

Atherogenesis:

The link between abnormalities of the coagulation system and
thrombosis of a coronary artery is well established not only
in terms of the actual clot but in terms of risk of myocardial
infarction in human populations. In many prospective studies,
the plasma levels or activities of coagulation factors such as
fibrinogen, factor VIIa and fibrin degradation products (D
dimer assays) have been shown to be predictive risk factors
for myocardial infarction, and other vascular events such as
stroke and progressive peripheral vascular disease (14, 15,
16). A substantial proportion of the risk due to cigarette
smoking is attributable to raised fibrinogen. The mode of
interaction of this risk factor with the actual lesions of
atherosclerosis, the atherosclerotic plaques within the artery
wall, remains unexplained, but the end result, accumulation of
fibrin admixed with the lipid core of the plaque and forming
overlying thrombus in minor and major terminal events is well
established.

The major pathogenic feature of atherogenesis is the response
to fibrin and lipid accumulation by the smooth muscle cells of
the arterial wall. Smooth muscle cell proliferation has long

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been recognised as the key event in plaque development, as it
is in the more acute lesion of post angioplasty restenosis
(17). Atherosclerotic lesions can be divided into several
types, the earliest thought to be the gelatinous lesions, the
5   precursors of the fibrous plaques. The early gelatinous
lesions contain little lipid but significant amounts of fibrin
related antigens (FRA) (18). In the more advanced lesions,
fibrin is deposited in layers suggesting repeated thrombotic
episodes (19). In all lesions the FRA are largely derived
10   from cross-linked fibrin not fibrinogen suggesting continuous
deposition and lysis of fibrin (20).

Soluble extracts of intima from active types of lesions from
human autopsy and surgical material have been shown by the
15   present inventors to stimulate cell proliferation in the *in
vivo* chick chorioallantoic membrane test model(21). This work
was extended to show that for a short series of stimulatory
extracts, the bulk of the activity was removed by passing each
through an affinity column containing antifibrin(ogen)
20   antibody (22). Selective removal was again achieved with a
bound specific anti fragment E antibody, but not with a bound
anti fragment D antibody.

Rheumatoid Arthritis:

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Rheumatoid arthritis is of unknown cause but is known to be
driven by the immune system. This causes episodic
inflammation of the synovial lining of the joint, with
deposition of fibrin which becomes organised by fibrovascular
30   ingrowth, termed pannus, forming a membrane rich in
inflammatory cells. This extends over the joint cartilage,
releasing proteolytic enzymes that digest and gradually
destroy the joint surface causing pain and immobility. Anti
inflammatory drugs help symptoms but do not arrest disease
35   progress significantly. Prevention of pannus extension during
acute episodes may well be advantageous.

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Diabetic retinopathy

Diabetic retinopathy is due to narrowing of the microvascular
blood supply vessels within the eye as elsewhere in the body
in poorly controlled diabetes mellitus. It is characterised
by proliferation of leaky new blood vessels in response to
ischaemic areas of retina. The combination of fibrovascular
proliferation and contraction involves the vitreous and
distorts and destroys the retina. Fibrin deposition and
degradation are likely to contribute to this disadvantageous
instance of normal healing and repair.

Renal Disease: Acute and focal glomerulonephritis are
characterised by deposition of both immune complexes and
fibrin in the glomerulus with associated inflammatory cells.
There is resultant cellular proliferation leading to
glomerulosclerosis and permanent loss of function with renal
failure in many cases. Anti-inflammatory drugs could be
supplemented with the agents to inhibit cell proliferation.

Tumour Growth and Metastasis:

For a malignant, invasive epithelial tumour of any type to
grow more than 1mm and invade surrounding normal tissues,
there is an absolute requirement for recruitment of a new
blood supply. This phenomenon of tumour angiogenesis provides
a target for therapeutic anti cancer intervention. These new
small capillary vessels are leaky, and plasma proteins
including fibrinogen are abundant in adjacent connective
tissue at the tumour edge. Although it was once believed that
fibrin was deposited at the moving edge of stroma surrounding
most invasive tumours as in a wound (23), it is now believed
that many tumour types do not display a complete set of
procoagulant factors for this to happen. However there are
two major exceptions, oat cell carcinoma of lung and clear
cell carcinoma of kidney (24, 25). These two tumour types are
common, are extremely vascular, show fibrin deposition, and

are hard to treat once spread by metastases from the site of origin has occurred. Some modest improvement in survival has been shown in a trial of terminally ill patients with oat cell carcinoma with the drug warfarin which inhibits clotting (26).

5   One problem with such drugs is that complete inhibition is never achieved because of very real risks of major haemorrhage. Inhibition of the fibrin E stimulatory contribution not to tumour growth but to tumour angiogenesis may be a useful adjunct to the current partially effective

10 treatments by chemotherapy and radiotherapy. Other tumour cells which may be used as a target include cells of solid tumours such as lung (including small cell lung), bowel (colon), breast, ovarian, prostate, stomach, liver, pancreatic and skin tumours, as well as leukaemias.

15

Fibrin Glues for Surgery: Modification of fibrin glues to promote or inhibit the cell proliferation induced by the fibrin degradation products can be achieved by preparation and admixture with promoters or inhibitors of the site of

20 interaction of fibrin E as provided by the present invention. These glues are increasingly used for a wide variety of operations where sutures are impractical.

In all these examples of disease, the clinical aim would be to

25 prevent the initiating cause but this is often not possible. Selective inhibition of angiogenesis and cell proliferation would be highly desirable. At the same time, it would be preferable to avoid interference with other aspects of the normal inflammatory response, and with blood clotting and

30 fibrinolysis.

However clinical manipulation at one site may conflict with clinical problems at other sites. For example, systemic administration of an antiangiogenic drug to inhibit tumour

35 growth may adversely affect normal healing and repair of a healing skin wound, or a peptic ulcer of stomach.

The severity of potential limitations depends on the
therapeutic margin of treatment success over unwanted side
effects. Short term treatment and localised treatment offer
solutions to some problems but not all. It is therefore

5 envisaged to utilise protective or therapeutic treatments at
the other sites and conditions at risk of collateral damage.
Therefore, treatments may be developed whereby cell
proliferation and/or angiogenesis inhibitors may be used at
the site of interest or systemically but locally protective

10 FDP induced cell proliferation and/or angiogenesis agonists
are provided at other sites that stimulate angiogenesis and
the normal cell proliferative response. Combinations of
systemic and local administration may become feasible and
effective.

15
In general, the methods will involve administering to a
patient in need of treatment an effective amount of a peptide,
analog or antibody (or composition thereof) of the invention.
Suitable routes of administration of compounds of the

20 invention include oral or parenteral, and will depend in part
upon the intended use and the discretion of the physician.
Small peptides may be administered orally although parenteral
administration may generally be more convenient in some
circumstances.

25
The amount of peptides, analogs or antibodies of the invention
administered to a patient is ultimately at the discretion of
the physician, taking account of the condition of the patient
and the condition to be treated.

30
Doses may be administered continuously, e.g in the form of a
drip, or at discrete intervals, e.g twice daily, daily, weekly
or monthly. Doses may also be administered topically to
achieve concentrations of active agent on the skin in the

35 ranges described above.

Where a peptide, analog or antibody of the invention is to be

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administered in conjunction with a cytotoxic or cytostatic
agent, the dose of said agent will be in accordance with
manufacturers' instructions.

5   Peptide, analogs or antibodies may be selectively directed to
tumour cells by various mechanisms in order to enhance their
effectiveness and to avoid effects on normal cells. Such
mechanisms include coupling the peptide, analog or antibody to
molecules which specifically interact with receptors or
10   antigens on target cells, such as VEGF receptors or CEA.
Alternatively gene therapy vectors expression peptides of the
invention may comprise an expression system whose promoter is
selectively activated in tumour cells, such as promoters
active in fetal liver cells.

15

In a further embodiment, a peptide, analog or antibody of the
invention may be incorporated into a stent which is introduced
into the arteries of a patient during an angioplasty
procedure. This is in order for the peptide, analog or
20   antibody of the invention to treat restenosis. The stent is a
hollow metal tube, usually made of stainless steel and
optionally coated with a polymeric material such as a plastic
which is expanded during the procedure so as to be left in
place in the artery to treat heart disease caused by arterial
25   narrowing. A problem with this procedure is the occurrence of
restenosis, i.e. the cardiovascular cells tend to grow back
and further treatment is ultimately required. By coating the
stent with a peptide, analog or antibody of the invention, the
peptide, analog or antibody is delivered locally into the
30   cardiovascular tissue and will prevent local regrowth of cells
by inhibiting stimulation of cell proliferation by FDPs.

Peptides, analogs or antibodies of the invention may be either
coated onto or incorporated into the stent by conventional
35   means known per se in the art. For example, the peptides,
analogs or antibodies may be mixed with a pharmaceutically
acceptable carrier compatible with the stent material and

coated on or into the stent. Where incorporation into the
stent is contemplated it is desirable that the stent comprises
an open celled polymeric structure. Where the stent is in the
form of a mesh, the peptides, analogs or antibodies may be
5   incorporated into a suitable delayed release carrier contained
in the spaces between the mesh strands. Delayed release
formulations are widely available for a number of different
purposes in the art; these include formulations based on
pharmaceutically acceptable polymers which dissolve slowly in
10   the body following implantation.

A number of coronary stents have been approved for clinical
use in the USA by the FDA. These include balloon expandable
stents such as the Palmaz-Schatz stent made by Cordis
15   Corporation (a division of Johnson & Johnson Interventional
Systems) and the Gianturco-Roubin II (GR-II) stent made by
Cook Cardiology (Bloomington, IN, USA). Self-expanding stents
are also used in the art, e.g. the Wallstent (Medinvent-
Schneider, Switzerland). Generally these stents are made of a
20   wire of around 0.1 mm (e.g. from 0.07 to 1.5 mm) diameter, are
designed to expand to a diameter of 3-5 mm, and are around 10
to 20 mm in length.

Examples of stent coatings to which reference may be made for
25   the provision of peptide coated stents of the invention
include a heparin-coated Palmaz-Schatz stent (Serruys *et al*,
Circulation, 1996, 93;412-422) and a platelet glycoprotein
IIa/IIIa receptor antibody polymer-coated stent (Aggarwal *et
al*, Circulation, 1996, 94;3311-3317).

30
For further guidance, those of skill in the art may also make
reference to "Coronary Artery Stents", an ACC Expert Consensus
Document (Pepine *et al*, J. Am. Coll. Cardiol., 1996, 28;782-
794.
35
The following examples illustrate the invention.

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Description of the Drawings.

Figure 1 illustrates the effect of admixture of 3 different rabbit anti phage antibodies with stimulatory FDP.

Figure 2 illustrates the effect of admixture of anti phage antibodies against selected phage clones 42, 43 and 44 to abolish the stimulatory effect of FDP. Student's t test on log transformed data shows a significant ($P<0.05$) increase in DNA synthesis compared with the buffer only control group by FDP. The 3 anti phage groups are significantly different from the FDP group, but not from the control group.

Figure 3 illustrates the effect of admixture of anti phage antibodies against selected phage clones 45 and 49 with stimulatory FDP. The stimulatory effect of FDP was inhibited by anti phage 45 ($P< 0.05$) but not with anti phage 49.

Figure 4 illustrates that peptide WTM110 inhibits the stimulatory effect of FDP.

Figure 5 illustrates that antibody raised to WTM abolishes the stimulatory effect of FDP

Examples of the Invention

Example 1

Fibrin fragment E binds to a 66kDa cell membrane component

i) Digoxygenin Labelling of Fibrin Fragment E

327mg of digoxygenin ester, dissolved in 8.18ml DMSO was added to 1mg of fibrin fragment E in 1 ml of phosphate buffered saline (PBS) and incubated at room temperature for 2hours. This was then dialysed against PBS (3 changes of 1l) to remove the unreacted ester and DMSO. Detection of the labelled

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protein was then carried out using anti-digoxygenin alkaline
phosphatase raised in sheep (Boehringer).

ii) Ligand blot

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Chick fibroblasts, Cos7, mouse 3T3 and human embryonic lung
cells (HEL) were cultured in a 25cm^2 flask (Nunc) until
confluent (approximately $3x10^6$ cells). The cells were then
floated off the flask using PBS with 2% EDTA and slight
10   scrapping with a rubber policeman. The cells were centrifuged
for 5min at 3,000rpm and resuspended in hypotonic shock
solution, sonicated for 30min and then centrifuged and
resuspended in 1ml of PBS. 100ml of the cells was then added
to 35ml of SDS containing gel loading dye (8%SDS, 40%glycerol,
15   12.5% 0.5M Tris-glycine buffer pH 6.8 and 10mg of bromophenol
blue), and heated to 95°C for 5min.

50ml of the cells in loading dye were applied to gradient
polyacrylamide gels (3-20%) and electrophoresed for 5 hours
20   until the dye reached the base of the gel. The gel was then
washed several times in Tris buffered saline containing 0.5%
Triton X-100 to remove the SDS. The gel was then blotted on
to PVDF membrane (Millipore) using a Bio-Rad blotting system
in Tris glycine buffer (25mM Tris and 192mM glycine).

25

The blotting membrane was blocked using 5% bovine serum
albumin in Tris buffered saline (TBS). The blot was then
incubated overnight at 37°C with digoxygenin labelled fibrin
fragment E (100mg in 100ml of TBS). The membranes were washed
30   three times in TBS 0.5% Tween 20 for 10 minutes and incubated
for 1 hour with sheep anti-digoxygenin antibody conjugated to
alkaline phosphatase. The membrane was washed, 3 times, again
with TBS Tween followed by development with nitro blue
tetrazolium, bromo-chloro-indolyl-phosphate in sodium
35   bicarbonate buffer (NaHCO$_3$ 100mM, MgCl$_2$ 10mM) to visualise the
position of the receptor containing band. For each cell type,
a band was identified of approximately 66 kDa. To confirm

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this finding, membrane immunoblot experiments were performed
using cells previously challenged with fibrin fragment E as
described below.

5   iii) Membrane Immunoblotting

Chick fibroblasts, Cos7, mouse 3T3 and human embryonic lung
cells (HEL) were cultured in a 25cm^2 flask (Nunc) until
confluent (approximately $3x10^6$ cells). 100mg of digoxygenin
10   labelled fibrin fragment E was added to the cells and
incubated at 37°C. Cells were harvested by rinsing with PBS
and then floated off the flask using PBS with 2% EDTA and
slight scraping with a rubber policeman. The cells were
centrifuged for 5min at 3,000rpm and resuspended in hypotonic
15   shock solution for 15 min, centrifuged and resuspended in 1ml
of PBS. 100ml of the cells was then added to 35ml of SDS
containing gel loading dye (8%SDS, 40%glycerol, 12.5% 0.5M
Tris glycine buffer, pH 6.8 and 10mg of bromophenol blue), and
heated to 95°C for 5min.
20
50ml of the cells in loading dye were applied to gradient
polyacrylamide gels (3-20%) and electrophoresed for 5 hours
until the dye reached the base of the gel. The gel was then
washed several times in Tris buffered saline containing 0.5%
25   Triton X to remove the SDS. The gel was then blotted on to
PVDF membrane (Millipore) using a Bio-Rad blotting system in
Tris glycine buffer (25mM Tris and 192mM glycine).

The blotting membrane was blocked using 5% bovine serum
30   albumin in Tris buffered saline (TBS). The blot was then
incubated overnight at 37°C for 1 hour with rabbit anti
fibrinogen antibody (Dako). The membranes were washed three
times in TBS 0.5% Tween 20 for 10 minutes and incubated for 1
hour with goat anti rabbit antibody conjugated to alkaline
35   phosphatase. The membrane was washed, 3 times, again with TBS
Tween followed by development with nitro blue tetrazolium
(NBT), bromo-chloro-indolyl-phosphate (BCIP) in sodium

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bicarbonate buffer (NaHCO$_3$ 100mM, MgCl$_2$ 10mM pH 9.8). This
resulted in a band of approximately 122kD, which is consistent
with fragment E (55kD) bound to a membrane fragment of 66kD.
Further evidence of binding to a receptor

iv) Cell Immunohistochemistry

Chick fibroblasts were cultured in a 25cm^2 flask (Nunc) until
confluent (approximately 3x10^6 cells). The cells were washed
in PBS and then trypsinised and diluted to 3x10^5 cells per ml
in Dulbecco modified medium with 10% foetal calf serum. 50ml
of the cells suspension was plated into each well of a Nunc
culture well slide. The cells were incubated overnight to
allow adhesion and recovery from the passaging. Digoxygenin
labelled fibrin fragment E was added to a final concentration
of 8mg per ml. Control wells contained unconjugated
digoxygenin and PBS.

The cells were rinsed after 2 hours with PBS then washed 3
times in TBS followed by one wash in distilled water. The
cells were then incubated for 2 hours with sheep anti-
digoxygenin alkaline phosphatase. The slides were then washed
three times in TBS and developed with NBT and BCIP in
bicarbonate buffer pH 9.8. The slides were air dried and
then mounted for observation of membrane staining under the
microscope. The cells were shown to bind fibrin fragment E.

v) Cell Binding Assays

Chick fibroblasts, Cos7, mouse 3T3 and human embryonic lung
cells (HEL) were cultured in a 25cm^2 flask (Nunc) until
confluent (approximately 3x10^6 cells). The cells were
trypsinised and resuspended at concentration of 1.3x10^5
cells/ml. 200μl of this cell suspension was added to each
well of a 96 well culture plate (Nunc). The cells were
incubated overnight at 37°C to allow adherence and recovery.
The cells were then divided into three sections, digoxygenin

controls, PBS controls and digoxygenin labelled fragment E
tests. Increasing concentrations of digoxygenin and
digoxygenin labelled fibrin fragment E were applied from 0-
30mg per well. The plates were incubated for 6 hours at 37°C
5 under culture conditions.

The plates were emptied of media and washed three times with
PBS. The cells were then incubated for 2 hours with sheep
antidigoxygenin alkaline phosphatase and developed with p-
10 nitrophenyl phosphate in bicarbonate buffer pH 9.8. The
plates were read on a Titertek Multiscan plate reader at
405nm. For each of the cell types, high absorbance was
demonstrated compared to the control preparations, indicating
binding of the digoxygenin labelled fibrin fragment E to the
15 cells.

The demonstration that fibrin fragment E binds to each of the
cell types tested suggests that a fibrin fragment E specific
receptor of 66kDa is present on the cell membrane.
20
Example 2

Antibodies to Fibrin Fragment E block FDP induced stimulation
of cell proliferation.
25
i) Polyclonal Antibody production

Polyclonal antibodies were raised to fibrin fragment E using
the following immunisation protocol.
30 Rabbits and rats were immunised with fibrin fragment E, made
by thrombin treatment of fibrinogen fragment E (Diagnostica
Stago). 50mg of fibrin fragment E in 0.5ml of PBS was mixed
with 0.5ml of Freunds complete adjuvant. The rabbits were
immunised intramuscular and the rats intraperitoneal. The
35 animals were then boosted at 4 and 8 weeks later with 50mg of
a long fibrin digest, which was shown to contain only fibrin
fragment E, in incomplete Freunds adjuvant. The animals were

then bled and the antisera tested for reactivity on blots of fibrin degradation products.

ii) CAM Cell proliferation assay

Cell proliferation was measured using the chick chorioallantoic membrane (CAM) model as described previously (4, 29, 30).The assay used is based on quantitative measurement of DNA synthesis in the CAM after exposure to control and test substances 18 h after application in liquid form to the whole "dropped" area of each CAM (4,29). This assay is a measure of changes in CAM vascularity (30).

The rabbit and rat antisera were mixed with active fibrin degradation products. These were applied to the chick CAM with a positive control (fibrin degradation products) and a negative control (the antibody alone). The CAM was processed for incorporation of tritiated thymidine. Results showed that the fibrin degradation products were active and that the rat and rabbit antisera to fibrin fragment E removed the activity.

Example 3

Identification of Peptides

The inventors have screened a peptide library made in Fd phage using polyclonal antisera capable of blocking activity of fibrin fragment E.

i) The Phage Library

Rat and rabbit polyclonal antibodies were raised against purified human fibrin fragment E as described previously (4). All secondary antibodies were purchased from commercial sources (Sigma Chemicals). A sample of the 2 x 108 clone 15 amino acid peptide gene VIII library as described by Scott & Smith (1990) (27), was obtained as a phage suspension from

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George Smith (University of Missouri Columbia, USA). 20 ml of
this phage suspension was infected into mid-log K91 cells,
which were IPTG induced when the OD reached approximately 0.9
resulting in the formation of 6.6 x 109 tetracycline-resistant
5   transductants. These were amplified by growth in one litre of
2 x TY medium containing 20 mg tetracycline/ ml for 24 hours
at 37°C with shaking.
Before precipitation of the phage, the cultures were first
spun for 10 minutes at 8,000 RPM at 4°C to remove bacteria.
10   The supernatant was kept, and PEG 6000 20% (w/v), 0.5 molar
NaCl, added resulting in a final PEG concentration of ~4%
(w/v). The cultures were then cooled on ice for 1 hour and
centrifuged for 20 minutes at 10,000 RPM at 4°C. The phage were
resuspended in 10ml of Tris buffered saline.
15

ii) Selection of Phage

Selection of phage from the library was carried out using a
procedure based on the biopanning method of Parmley & Smith
20   (1988) (9). 1 ml of each polyclonal antibody in PBS at
dilutions from 1: 200 to 1: 1,000 was added to a 60 mm petri
dish (Nunc) and incubated in an orbital incubator at room
temperature overnight to allow antibody adhesion. The next day
5mls of blocking solution (PBS, 0.5% Tween, 5 % skimmed
25   milk), was added to each dish, in order to block any sites not
adhered to by the polyclonals. The dishes were then left to
incubate in an orbital incubator for 1 hour at room
temperature, and then washed 5 times in PBST (PBS, 0.5%
Tween).
30

100μl of the gene VIII library was added to each dish, and the
dishes incubated at room temperature for 1 hour, in an orbital
incubator. The unattached phage were then poured away and the
dishes washed 5 times in PBS-Tween. Phage which remained
35   bound to the immobilised polyclonals were recovered from the
antibodies by the addition of 800μl 0.1 M HCl (pH. 2.2 with
glycine), and allowed to incubate at room temperature for 15

minutes.   48μl of unbuffered 2 M Tris base was then added to
each dish to neutralise the acid.

For round 1 biopanning, the contents of each dish were added
5   to 3ml cultures of mid-log E. coli K91, and allowed to
incubate at room temperature for 10 minutes.   12 ml of LB
medium (10g Tryptone, 5g yeast extract, 10g NaCl in 1 litre
H_2O, pH 7.5) was then added to each culture.   Tetracycline was
also added to a concentration of 0.2 mg/ml, and the cultures
10   incubated at 37°C (225 RPM) for 40 minutes.   Additional
tetracycline was then added, to a final concentration of 20
mg/ml and the cultures allowed to grow for 16-20 hours at 37°C
(225 RPM). Phage input and output titres were determined by
plating and incubation on LB agar dishes.
15

A second round of amplification was carried out as for the
first, except that 100μl of phage recovered from the first
rounds of biopanning was used against the blocked polyclonals.
A third round of biopanning was also carried out except that
20   100μl of phage recovered from the second round of biopanning
was used against the blocked polyclonals.

During rounds 2 & 3 negative controls, consisting of K91
cells, with no added phage, were used to demonstrate that the
25   K91 stocks had not acquired tetracycline resistance by another
means i.e. plasmid transfer. A fourth round of amplification
was carried out as for the third except that 100μl of phage
recovered from the second round of biopanning was used against
polyclonals from a different species, i.e. phage recovered
30   after three rounds of biopanning against the rabbit
polyclonals, was used against the rat polyclonals, and phage
recovered after three rounds of biopanning against the rat
polyclonals was used against the rabbit polyclonals. This was
to demonstrate the homogeneity of different polyclonals raised
35   in two different species against the same antigen and to
reject non shared target epitopes.

41

iii) Testing of Reactive Clones

Reactive clones were identified from the output of round 4
with an ELISA using a 1: 200 dilution of rat anti fibrin E
5 polyclonal for coating one set of wells, and the second coated
with a 1: 200 dilution of rabbit anti fibrin E polyclonal.
Samples of the selected phage clones were each run on PAGE and
immunoblotted with the existing anti E antisera that were the
basis of the selection process. The 60 selected reactive
10 clones were combined into 3 groups, 1-20, 21-40, and 41-60,
and samples mixed with Freund's complete adjuvant and used to
immunise rats and rabbits. The resultant new antisera were
tested initially by immunoblotting of PAGE of selected phage
clone proteins and also human FDP. Polyclonal antisera raised
15 against 21 to 40, and 41 to 60 detect fragment E bands on
immunoblots. In contrast, polyclonal antisera raised against 1
to 20 do not.

The rabbit antisera were then tested for ability to block the
20 stimulatory activity of human FDP on the chick CAM model. The
assay used was based on quantitative measurement of DNA
synthesis in the CAM after exposure to control and test
substances 18 h after application in liquid form to the whole
"dropped" area of each CAM (4,29). This assay is a measure of
25 changes in CAM vascularity (30). Anti phage antisera, raised
by relatively short term immunisation of rabbits, were used at
a ½ dilution and admixed with FDP used at a concentration of
approximately 1.95 mg/ml diluted before use to 1/10. Controls
included buffer only and antiserum only groups of CAMs. After
30 filter sterilisation, 0.3 ml was added onto each CAM dropped
surface.

iv) Demonstration of Blocking of FDP Angiogenic Activity

35 Figure 1 illustrates one experiment where rabbit anti phage 1-
20 does not appear to block the stimulatory effect of FDP
after admixture, but rabbit anti phage 21-40 and 41-60 do

42

inhibit the stimulation. Repeat experiments have now been completed a further 2 times for the latter two antibodies with similar inhibition. Antibody alone and buffer alone controls have no effect.

5

v) Testing of individual clones

Antibodies raised against individual clones 41-60 were tested for inhibitory activity using the chick CAM assay. Antibodies
10 raised against clones 42, 43, 44 and 45 were found to be inhibitory (Figs 2,3). In contrast, antibodies to clones 49 (Fig 3) and 53, for example, were not inhibitory.

vii) Sequencing of individual peptides
15

Amino acid sequences were obtained for clones 45 and 49. Peptides recognised by clone 45 antiserum were obtained by re-screening the phage library. The following four peptide sequences were obtained for clone 45:

20

CRAHSFGSPRPLPVV   (SEQ ID NO:1)
SRAHSFGSPRPLPVV   (SEQ ID NO:2)
CRAHSFVSPRPLPVV   (SEQ ID NO:3)
QPDPHLMMWKLPGFP   (SEQ ID NO:4)

25

A single sequence was obtained for clone 49
ALSKRPVGRPRVCTG   (SEQ ID NO:5)

Example 4

30

i) Peptides corresponding to the amino acid sequences of clone
45 inhibit cell proliferation.

The peptide corresponding to SEQ ID NO:1 was synthesised and
35 named WTM110. WTM110 was tested for modulation of FDP induced cell stimulation using the chick CAM assay as described above. The results are shown in Figure 4. At 7.5µg/ml, there is

little inhibition of cell proliferation. However, when the
concentration was increased to 15 μg/ml, inhibition was
demonstrated with cell proliferation reduced to control level.

5   ii) Antibody raised to WTM110 inhibits cell proliferation.

An antibody was raised to WTM110 and used in the chick CAM
assay as described above. As shown in Figure 5, in the
presence of anti-WTM110 antiserum, stimulation of cell
10 proliferation by FDPs was inhibited.

The sequence information for the finally selected epitopes,
derived from analysis of each phage clone DNA insert, can be
used not only to locate the active site on the molecule, and
15 to perpetuate blocking antibodies, but also to synthesise
large quantities of short peptides and short peptide
analogues. These can be tested for competitive blocking
activity for human fragment E. Such peptides and analogs are
potential therapeutic agents in the longer term for blocking
20 the cell stimulatory effects of fibrin fragment E in vivo in a
potentially wide variety of pathologies (30) without the
attendant risks of interfering in clotting or fibrinolysis.
Our previous work has shown that admixture of blocking
antisera to fibrin E will inhibit the angiogenic effect of
25 experimental mouse wound extracts (31) and extracts of
proliferative types of human atherosclerotic plaques (21). We
have not yet attempted inhibition in vivo. Sustained delivery
in vivo to inhibit, for example, wound healing should be more
readily achieved by administration of small peptides than
30 polyclonal antisera from another species.

The sequence information for the finally selected epitopes,
derived from analysis of each phage clone DNA insert, has been
used to locate parts of the active site on the molecule. The
35 sequence information can also be used to synthesise large
quantities of short peptides. These can be tested for
competitive blocking activity for human fragment E. Such

44

peptides are potential therapeutic agents.

Knowledge of the location of the active site, even though limited to a few short segments of the protein molecule,
5 allows further exploration of non-immunogenic adjacent areas, within the known molecular structure of fibrin E, which may influence biological activity. Synthetic peptides and analogs of such regions are likely to provide further experimental agents that are potential drugs.
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